Final Report

Original 2 of 2

Evaluation of [redacted]
in the BCOP Test
following OECD Guideline 437 dated [redacted]

Study No.: [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

Monitor:
[redacted]

Study Director:
[redacted]

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1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 in the version of 02 July 2008 published in Bundesgesetzblatt No. 28/2008, pp. 1146 - 1184

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

[redacted]

[redacted]
Date

Information on Study Organisation:

[redacted]

Deputy Study Director

Study Plan dated

Experimental Starting Date

Experimental Completion Date

Draft Report dated

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.
Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.
The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.
Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.
The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	[redacted]		
Performance of study			
Draft report			
Final report			

[redacted]

Date [redacted]

Quality Assurance Manager

Table of Contents

3 SUMMARY

Title of Study: Evaluation of [redacted] in the BCOP Test following OECD Guideline 437 dated [redacted]

Findings and Results:

One valid experiment was performed.

Bovine corneas were used. They were collected from slaughtered cattle which were between 12 and 60 months old.

The test item [redacted] was brought onto the cornea of a bovine eye which had been incubated with cMEM without Phenol red at 32 ± 1°C for one hour and whose opacity had been measured. The test item was incubated on the cornea for 4 hours at 32 ± 1°C. After removal of the test item and two hours post-incubation, opacity and permeability values were measured.

Physiological sodium chloride solution was used as negative control. The negative control showed no irritating effect on the cornea.

20% Imidazole was used as positive control. The positive control induced a severe irritation on the cornea.

The test item [redacted] showed no effects on the cornea of the bovine eye. The calculated IVIS (in vitro irritancy score) is 0.0160.

In conclusion, it can be stated that in this study and under the experimental conditions reported, the test item [redacted] possesses no eye irritation potential.

4 PURPOSE OF THE STUDY

This "in vitro" study was performed to assess the corneal irritation and damage potential of [redacted] by quantitative measurements of changes in opacity and permeability in a bovine cornea. The study was performed for regulatory purposes.

Sponsor's intent: REACH.

5 LITERATURE

The study is conducted in accordance with the following guideline:

- OECD Guideline for the Testing of Chemicals No. 437: "Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants" edition adopted 07 Sept. 2009

Additional information was taken from:

- "The Bovine Corneal Opacity and Permeability Assay", INVITTOX (UK) protocol no. 98, dated February 1994
- "Bovine Corneal Opacity and Permeability (BCOP) Assay", SOP of Microbiological Associates Ltd., UK, Procedure Details, April 1997; Gautheron et al. (1992), refined by Vanparys et al. (1994)

[redacted]

6 MATERIALS AND METHODS

6.1 Test Item

6.1.1 Specification

The following information concerning identity and composition of the test item were provided by the sponsor.

Name
Batch no
Appearance
Composition

CAS-No.
EINECS-No.
Molecular formula
Molecular weight
Purity
Homogeneity
Vapour pressure
Stability
Solubility
Production date
Expiry date

[redacted]

Storage	Room Temperature (20 ± 5 °C)
Hazard information	not stated
R-phrases	none stated
S-phrases	none stated

6.1.2 Storage
The test item was stored in the test facility at room temperature.

6.1.3 Preparation
The test item is a [redacted] Since no solution was feasible, it was tested directly, without dilution or preparation of a solution.

6.2 Positive Controls

The following substance was used as positive control:
For [redacted] test items, diluted as well as undiluted, in the „open chamber method":
20% imidazole solution; $C_3H_4N_2$, CAS-No. 288-32-4, dissolved in 0.9% sodium chloride solution, 20% solution (20 g/100 mL)

6.3 Negative Control

6.3.1 Sodium Chloride Solution
NaCl, 0.9% solution in deionised water, CAS-No. 7647-14-5

6.4 Test System

6.4.1 Specification
Species fresh bovine corneas

6.4.2 Origin
Freshly bovine eyes were obtained from the slaughterhouse Müller Fleisch GmbH, Enzstr. 2.4, 75217 Birkenfeld, Germany, on the day of the test. The cattle were between 12 and 60 months. The eyes were transported to the test facility in Hank's balanced salt solution (supplemented with 0.01% streptomycin and 0.01% penicillin). Then the corneas were dissected and incubated with media at 32 ± 1°C in an incubation chamber for 1 hour.

6.5 Chemicals

- Incubation Medium MEM (Minimum Essential Medium) without phenol red, Supplier Gibco/Invitrogen
- Incubation Medium MEM (Minimum Essential Medium) with phenol red, Supplier Gibco/Invitrogen
- Sodium bicarbonate $NaHCO_3$, Supplier Merck
- L-glutamine, supplier: Biochrom AG
- 1% fetal calf serum, supplier PAN BioTech (EU approved)
- cMEM without phenol red: 495mL MEM are supplemented with 1.1 g $NaHCO_3$, 5 mL FCS and 4.57 g L-Glutamine.
  (As soon as the MEM without phenol red is supplemented with $NaHCO_3$, FCS and L-Glutamine, it is designated in the following as cMEM (= complete MEM) without phenol red.)
- cMEM with phenol red: 495 mL MEM with phenol red are supplemented with 1.1 g $NaHCO_3$, 5 mL FCS and 4.86 g L-Glutamine.
  (As soon as the MEM with phenol red is supplemented with $NaHCO_3$, FCS and L-Glutamine, it is designated in the following as cMEM (= complete MEM) with phenol red.)
- Fluorescein-Na
- Hank's Balanced Salt Solution (HBSS), supplier: Life Technologies
- 1% Penicillin-Streptomycin solution, supplier: PAN Biotech

6.6 Test Vessels

All vessels used are made of glass or sterilizable plastic. They were sterilised before use by heating to 180 °C (two hours) or autoclavation.

The following vessels were used:

- Schott-bottles, glass vials, and culture flasks for solutions and media

6.7 Instruments and Devices

The following instruments and devices were used in the performance of the study.

- Autoclave MLS 3020
- Tweezers
- Scalpel
- Scissors
- Screwdriver
- Syringes (5mL, 10mL, 20mL) with needles
- vacuum pump for evacuating the media
- Stop watch
- Water bath GFL, 32 ± 1°C
- Corneal chambers
- Fuse tongs
- Scales Mettler PB 5001-S02
- Analytical scales XS 205 DU
- Spectral photometer Specord 205, Analytik Jena
- Incubation chamber No. 10, Memmert, 32 ± 1°C
- Adjustable pipettes [redacted] (200-2000µL) and [redacted] (20-200µl) with sterile tips
- weight board, mortar & pestle
- Clean bench AXOSafe Mars
- Pipetting device Accu Jet
- Glass Thermometer 2005 0117-73; 2002 0912-22

Usage and, if applicable, calibration following the corresponding SOP in the current edition. Standard laboratory material (e.g. glassware) was also used.

7 CONDUCT OF THE STUDY

7.1 Preparations

After having carefully cleaned and sterilised the corneal holders, they were kept in the incubation chamber at 32°C.

On the day of the assay, the MEM without Phenol red was supplemented with sodium bicarbonate, L-glutamine and 1% fetal calf serum (= complete MEM) and stored in a water bath at 32°C ± 1°C.

The same was performed with the MEM with Phenol red.

After the arrival of the corneas they were examined and only corneas which were free from defects were used. The corneas were excised with a scalpel and cut from the globe with a 2-3 mm ring of sclera around the outside. Each cornea was transferred to a corneal holder in which pre-warmed cMEM without Phenol red was filled. The holders were then incubated for one hour in the incubation chamber at 32°C.

7.2 Experimental Parameters

Date of treatment	[redacted]
Incubation time	4 hours
Positive controls	imidazole solution, 20%

7.3 Method Description

After the initial incubation, the medium was changed and the baseline opacity for each cornea was recorded. None of the corneas showed tissue damage; therefore, all corneas were used.

The baseline opacity was measured by placing the holder with the cornea in a spectrophotometer and recording the absorption at 570 nm. Opacity is calculated from the measured absorption following the equation stated in chapter 8, page 13.

For each treatment group (negative control, positive control and test item), three replicates were used. 750 µl negative control resp. an appropriate amount of test item resp. positive control solution were applied to each replicate.

The test item and the controls were applied in the same manner.

According to the characteristics of the test item, the following treatment procedure was performed:

7.3.1 Open Chamber Method

The "open chamber-method" is used for non-surface-active solids.

In order to apply the test item, the nut was unscrewed to remove the glass disc. The test item could be applied directly on the cornea now.

In average, 231.4 mg of the test item were tested neat and applied directly on the cornea using a weight board. The test item was given on the epithelium in such a manner that as much as possible of the cornea was covered with test item.

Exposition time on the corneas was 4 h ± 5 min. at 32°C. After thorough rinsing with cMEM with phenol red and final rinsing with cMEM without phenol red, both chambers were filled with cMEM without phenol red, and the final opacity value of each cornea was recorded at once (again by measurement at 570 nm). The cMEM without Phenol red was then re-

moved from the front chamber, and 1 mL sodium fluorescein solution (concentration 5 mg/mL) was added to the front chamber.

The chambers were then closed again and incubated for 90 ± 5 min at 32 ± 1 °C. After incubation, the content of the posterior chamber was thoroughly mixed. Then, the permeability was measured with the spectral photometer as optical density at 490 nm.

8 EVALUATION

8.1 Calculation of Opacity value

Opacity is calculated from the measured absorption at 570 nm following

$O = 1 / 10^{(-A)}$

with

O = Opacity

A = Absorption at 570 nm

8.2 Correction of Measured Absorption at 490 nm

As cuvettes with a pathlength of 0.2 cm were used in the measurement of the Fluorescein-Na solution in the spectrophotometer, the pathlength must be corrected to 1 cm.

Coefficient: 1/0.2 = 5: all absorptions are multiplied with this coefficient.

8.3 Calculation of IVIS (in vitro irritancy Score)

The IVIS of the negative control was calculated from the following equation:

$$IVIS = \text{mean opacity value} + (15 \times \text{mean } OD_{490} \text{ value})$$

The IVIS of the positive control and of the test item is calculated from the following equation:

$$IVIS = (\text{opacity} - \text{opacity}_{\text{negative control}}) + [15 \times (\text{corr. } OD_{490} - \text{mean corr. } OD_{490\text{negative control}})]$$

9 FINDINGS AND RESULTS

9.1 Opacity and Permeability Values

The absorption (570 nm) and opacity values which were measured before and after exposition are given in the following table:

Table 9.1-a Absorption and Opacity values [redacted]

Parameter	Negative Control						Positive Control		
Absorption before exposition	0.1667	0.2072	0.2001	0.3220	0.2749	0.3070	0.3004	0.2663	0.2660
Absorption after exposition	0.4163	0.3447	0.3923	0.3948	0.3812	0.5072	1.7375	1.7279	1.7893
Opacity before exposition	1.4678	1.6114	1.5852	2.0988	1.8832	2.0276	1.9969	1.8464	1.8450
Opacity after exposition	2.6077	2.2117	2.4675	2.4818	2.4056	3.2151	54.6388	53.4454	61.5609
Opacity Difference	1.1399	0.6003	0.8823	0.3830	0.5224	1.1875	52.6419	51.5990	59.7159

Mean opacity difference of the negative control is 0.8742.

For the permeability measurement, three replicates for each cornea were measured. The optical density values at 490 nm are given in the following table:

Table 9.1-b Optical density at 490 nm

Repl.	Negative Control			Safire 400			Positive Control		
1	-0.0030	-0.0040	-0.0040	-0.0010	-0.0030	0.0010	0.210	0.335	0.327
Net	-0.0137	-0.0220	-0.0205	-0.0067	-0.0152	0.0044	1.0501	1.6753	1.634
Mean	-0.0187			--					
Net – Neg. Contr.				0.0120	0.0035	0.0231	1.0688	1.6940	1.653

In order to correct the path length, a factor of 5 was taken into account when calculating the IVIS (see following page). Then, from all absorption values, the net mean absorption of the negative control (-0.0187) was subtracted from the individual replicates of test item and positive control.

9.2 IVIS Values

IVIS was calculated using the values in tables 9.1-a and 9.1-b and the equation stated in chapter 8.3.

Example:

IVIS [redacted] Repl. 1) = (0.3830 – 0.8742) + [15 * (5 * -0.001 – (-0.0187))] = -0.3112

The calculated IVIS for each replicate and the corresponding means are presented in the following table:

Table 9.2-a IVIS

Test Group	IVIS	Mean IVIS	Relative Standard Deviation IVIS
Negative Control 0.9% NaCl	0.9344	0.5930	56.1%
	0.2703		
	0.5748		
[redacted]	-0.3112	0.0160	3482.5%
	-0.2993		
	0.6598		
Positive Control 20% Imidazole	67.7997	75.8590	10.4%
	76.1348		
	83.6427		

Note: the high relative standard deviation of the IVIS of test item and negative control is due to mathematical reasons, as the respective means are very small.

9.3 Classification

Classification of the irritation potential is made as stated in the following table:

Table 9.3-a Classification

In vitro irritancy score (IVIS)	Classification
0 - 3	Non eye irritant
3.1 - 25	Mild eye irritant
25.1 - 55	Moderate eye irritant
55.1 - 80	Severe eye irritant
≥ 80.1	Very severe eye irritant

Classification was derived from Gautheron et al. (1992), refined by Vanpaarys et al. (1994) and confirmed by ICCVAM.

In the negative control, no signs of eye irritation were observed.

The positive control showed severe eye irritation.

The test item [redacted] showed no eye irritation (values lay below negative control).

9.4 Validity

According to the guideline, the test is considered as valid if the positive control causes an IVIS that falls within two standard deviations of the current historical mean (at least the last three months).

The negative or solvent control has to show an IVIS between 0 and 3.

The validity criteria and findings are given in the following table:

Table 9.4-a Validity

Parameter	Criterion	Found	Assessment
IVIS of negative control 0.9% NaCl	0 - 3	0.5930	ok
IVIS of positive control 20% Imidazole	38.6 – 128.6	75.8590	ok

Values for positive control were within the range of historical data of the test facility (see 14, page 19). Therefore, the test system was acceptable.

10 Discussion

This in vitro study was performed to assess the corneal irritation and damage potential of [redacted] by quantitative measurements of changes in opacity and permeability in a bovine cornea.

The test item [redacted] was brought onto the cornea of a bovine eye which previously had been incubated with cMEM without Phenol red at 32 ± 1°C for one hour and whose opacity had been determined. The test item was incubated on the cornea for 4 hours at 32 ± 1°C. After removal of the test item and two hours post-incubation, opacity and permeability values were measured.

Physiological sodium chloride solution was used as negative control, imidazole (20% solution in 0,9% sodium chloride solution) was used as positive control.

The positive control induced a severe irritation on the cornea, mean IVIS was 75.8590.

The negative control showed no irritation, mean IVIS was 0.5930.

The test item was tested pure. A mean IVIS of 0.0160 was calculated, corresponding to a classification as not eye irritant.

No observations were made which might cause doubts concerning the validity of the study outcome. The test is considered valid.

11 DEVIATIONS

11.1 Deviations from the Study Plan

No deviations from the study plan were observed.

11.2 Deviations from the Guideline

None as known.

12 RECORDING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for fifteen years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for fifteen years; then, the retain sample will be discarded.

Number of originals which will be sent to the sponsor: 1

13 ANNEX 1: COPY OF GLP CERTIFICATE

RheinlandPfalz

Gute Laborpraxis / Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gem. /according to § 19 Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/ EEC at::

Prüfeinrichtung / Test facility

[redacted]

Prüfung nach Kategorien / Areas of Expertise
(gem. / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8

Datum der Inspektion / Date of Inspection
(Tag.Monat Jahr / day.month year)
27. und 28. November 2006

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.
Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist so rechtzeitig zu beantragen, dass die Folgeinspektion spätestens vier Jahre nach dem Beginn der o.g. Inspektion stattfinden kann. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit:

Based on the inspection report it can be con- firmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Priciples of the GLP has to be applied for in time to allow for a follow-up inspection to take place within four years after commencing the above mentioned inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date

11.01.2007

Dr.-Ing. Karl-Heinz Rother - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht 2 Rheinland-Pfalz Siegel

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7
55116 Mainz

(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht

[redacted]

14 ANNEX 2: COMPARISON WITH HISTORICAL DATA

In the following table, the means of the negative controls and positive controls of all performed experiments up to [redacted] (before finalisation of the study plan) is stated and compared with the values which were found in this study.

14.1 Historical Data of Negative and Positive Control

Table 14.1-a Historical Data

Parameter	IVIS Negative control	IVIS Positive control
Substance	0.9% sodium chloride solution	Imidazole solution 20%
Mean	1.155	83.6
Standard Deviation	0.723	22.5
Range (min – max)	0.1328 – 2.2813	43.399 – 132.4131
Range (Validity)	-	38.6 – 128.6
Study [redacted]	0.5930	75.8590